

OBSERVATIONS ON *SPIRRILLUM SPUTIGENUM* AND ITS RELATIONSHIP TO *SELENOMONAS* SPECIES WITH SPECIAL REFERENCE TO FLAGELLATION¹

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Two strains of *Spirillum sputigenum* from the oral cavity were described in detail by Macdonald (1953). The organisms were gram-negative and appeared as either curved or S-shaped spirillar rods from 4 to 50 μ long and were apparently peritrichously flagellated. Macdonald (1953) recommended that these forms be included in Bergey's classification as a recognized species of the genus *Spirillum*, and be listed as (a) one micron or less thick, (b) no volutin granules observed, and (c) having peritrichous flagella. In 1954 Lessel and Breed recommended the recognition of the genus *Selenomonas* and the inclusion of *Spirillum sputigenum* as one of three species in this genus under the name of *Selenomonas sputigena*. The other two species were *Selenomonas palpitans*, from the intestinal tract of the guinea pig, related rodents, and herbivorous mammals, and *Selenomonas ruminantium*, from the rumen juice of domestic animals. The former was described first by von Prowazek (1913) and named by Simons (1921). The latter was described by Certes (1889) as a protozoan species, *Ancyromonas ruminantium*; Wenyon (1926) renamed it *Selenomonas*. This organism was isolated in pure culture by Huh-tanen and Gall (1953) and by Bryant and Small (1956). Bryant (1956) described a number of physiological characteristics of several strains and noted that production of H₂S by *Selenomonas ruminantium* was the only known feature distinguishing it from *Spirillum sputigenum*.

Each of the three organisms has been described by one or more authors as having a flagellum or tuft of flagella attached near the middle of the concave side (Hoffman and von Prowazek, 1906; von Prowazek, 1913; Boskamp, 1922; Certes, 1889; Kerandel, 1909; Woodcock and Lepage,

1913; Robinow, 1954). It is on this basis that the three have been related by Lessel and Breed.

A satisfactory method for the isolation of *Spirillum sputigenum* from the oral cavity has been described recently (Macdonald and Madlener, 1957). The method involved the use of a medium of veal heart infusion, sodium lauryl sulfate, and sheep's serum. Material obtained from the gingival crevice of humans was streaked on plates of this medium and incubated in a hydrogen atmosphere. From these plates *Spirillum sputigenum* was recovered frequently in pure culture. The significant features of the method were that *Spirillum sputigenum* grew as a surface film, sodium lauryl sulfate inhibited part of the oral flora which otherwise tended to overgrow *Spirillum sputigenum*, and sheep's serum permitted an essential rapid drop in Eh of the surface layers of the medium in a Brewer jar.

The present report dealing with 11 strains of *Spirillum sputigenum* confirms the description of this organism by Macdonald (1953) and examines the question of flagellation and the relationship of *Spirillum sputigenum* to *Selenomonas palpitans* and *Selenomonas ruminantium*.

METHODS

Eleven strains of *Spirillum sputigenum* were isolated from the human oral cavity using sodium lauryl sulfate-sheep serum agar (Macdonald and Madlener, 1957). They were examined for production of indole and hydrogen sulfide, reduction of nitrates, fermentation of glucose, sucrose, and mannite, production of acid in litmus milk, and pH range for the support of growth, according to methods previously described (Macdonald, 1953).

The potential required to permit initiation of growth was investigated in heart infusion broth (Difco) with 1 per cent added glucose, dispensed

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in 6.0 ml lots. The fall in potential of this medium in a Brewer hydrogen jar was recorded at 45 min, 1 hr, and half-hourly thereafter for 6 hr. Measurements were made with a Beckman model G potentiometer with platinum electrode and agar bridge inserted into the medium immediately following removal from the jar. Concurrently, in each jar four additional tubes of medium were reduced. These were covered promptly with 8 cm of sterile paraffin oil when the jars were opened. Each tube was then inoculated with 0.1 ml of a 48-hr culture of a strain of *Spirillum sputigenum* grown in agar-free thioglycolate broth, using a different strain for each tube. All tubes were incubated aerobically for 48 hr. Positive controls consisted of 0.1 ml inoculations of *Spirillum sputigenum* into heart infusion-thioglycolate broth similarly covered with oil and incubated aerobically.

Tests of pathogenicity were carried out using the methods described by Macdonald (1953). Six strains in thioglycolate broth (Difco) were inoculated subcutaneously in 1.0-ml amounts into guinea pigs (1 guinea pig per strain); intraperitoneally in 0.2-ml amounts into mice (2 per strain); intravenously into rabbits (0.5-ml amounts, 1 rabbit per strain; 1.0-ml amounts, 1 rabbit each for 2 strains only). The guinea pigs and mice were observed daily for 7 days and then sacrificed.

All 11 strains were examined by Gram stain, Giemsa stain, and by darkfield examination, using very young cultures (6 hr) and older cultures (24 hr to 6 days).

Several strains were examined by electron microscopy. Twenty-four or 48-hr thioglycolate broth cultures were fixed with 2 per cent osmium tetroxide, and centrifuged and washed in distilled water three times. Screens were examined unshadowed or shadowed with palladium. Two strains of *Selenomonas ruminantium*, kindly provided by Dr. Marvin P. Bryant, Dairy Husbandry Research Branch, United States Department of Agriculture, Beltsville, Maryland, were grown for 48 hr in thioglycolate broth (Difco) and prepared for electron microscopy in the same way.

Preparations of the contents of guinea pig ileum were examined by darkfield for *Selenomonas palpitans*. Forms morphologically similar to but larger than *Spirillum sputigenum* were regularly seen. Unsuccessful attempts were made to culti-

vate this organism. Sodium lauryl sulfate-serum agar was used (Macdonald and Madlener, 1957), but the plates invariably were overgrown with *Proteus* species. Rabbit antiserum prepared against 3 *Proteus* strains was incorporated in the medium. It inhibited the spread of *Proteus* but *Selenomonas* did not grow. *Spirillum sputigenum* strains added to the contents of guinea pig ileum could be recovered regularly in pure culture.

RESULTS

All 11 strains of *Spirillum sputigenum* conformed in their characteristics to the previous description of two strains (Macdonald, 1953). They failed to produce indole or hydrogen sulfide; they reduced nitrate, fermented glucose, sucrose mannite, and litmus milk; and grew over a pH range of 4.5 to 8.6.

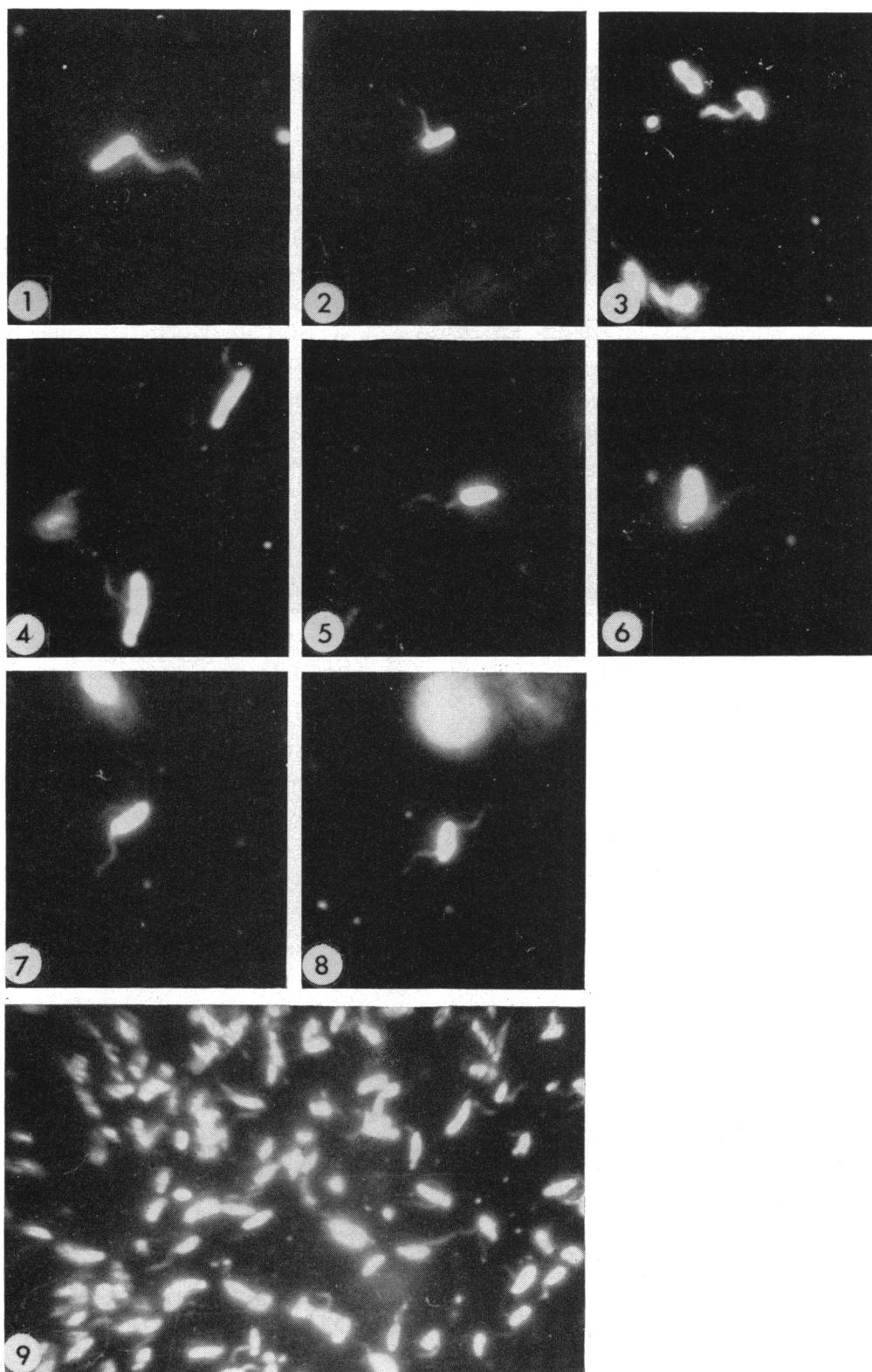
Four strains tested grew in glucose heart infusion broth which had been reduced in a hydrogen jar for 1 hr. None grew if the medium had been reduced for only 45 min. The potential of the medium after 1 hr in a hydrogen jar was -50 mv (calculated to the normal hydrogen electrode). The potential after only 45 min in the jar was -35 mv.

None of the guinea pigs or mice injected with cultures of *Spirillum sputigenum* showed any signs of infection or toxicity during the 7-day observation period. All appeared normal at autopsy.

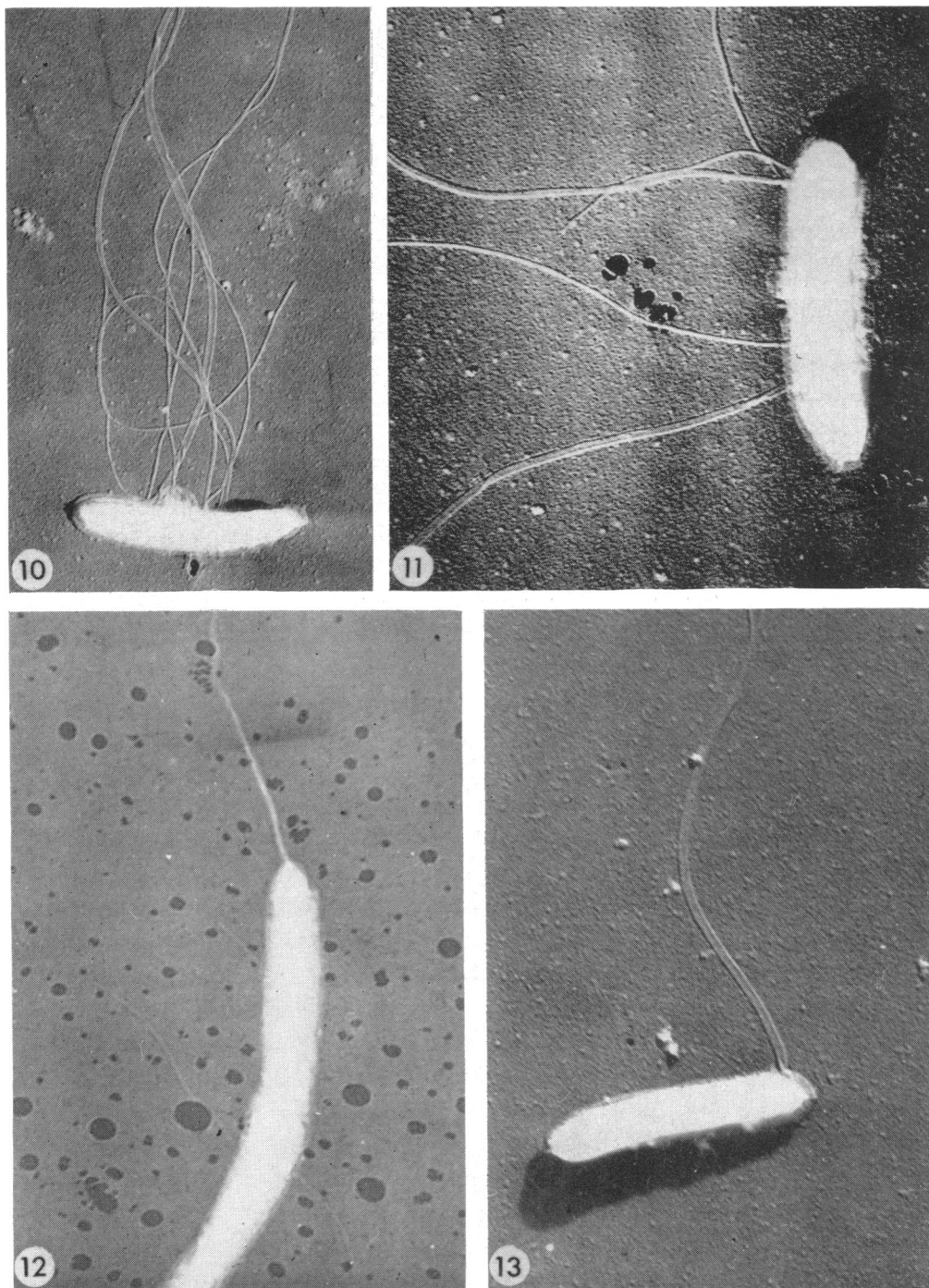
All the injected rabbits showed increased heart rate, dyspnea, and hyperpnea within 10 min of inoculation. Two animals inoculated with 1.0 ml of culture died in 4 hr, and two inoculated with 0.5 ml died in 12 hr. The former two animals were inoculated with strains which were not lethal in a dosage of 0.5 ml. Death in each instance was preceded by hindquarter flaccid paralysis. Post-mortem examinations showed distention of the large bowel, absence of peristalsis, and congestion of liver portal areas and lungs as described by Macdonald (1953). Four rabbits survived and appeared normal at autopsy after 7 days.

The 11 strains of *Spirillum sputigenum* were gram-negative. Using the Giemsa technique (Wellings, 1938) the cells stained pink to pale violet with irregularly distributed darker violet granules.

By darkfield examination, cells from 48-hr



Figures 1-9. Darkfield photomicrographs of strains of *Spirillum sputigenum*, in thioglycolate broth, photographed at magnification $\times 1000$.



Figures 10-13. Electron photomicrographs of strains of *Spirillum sputigenum* photographed at magnification $\times 5900$ ($\times 5369$ in reproduction here). Figures 10, 11, and 13 are shadowed with palladium. Figure 12 is unshadowed.

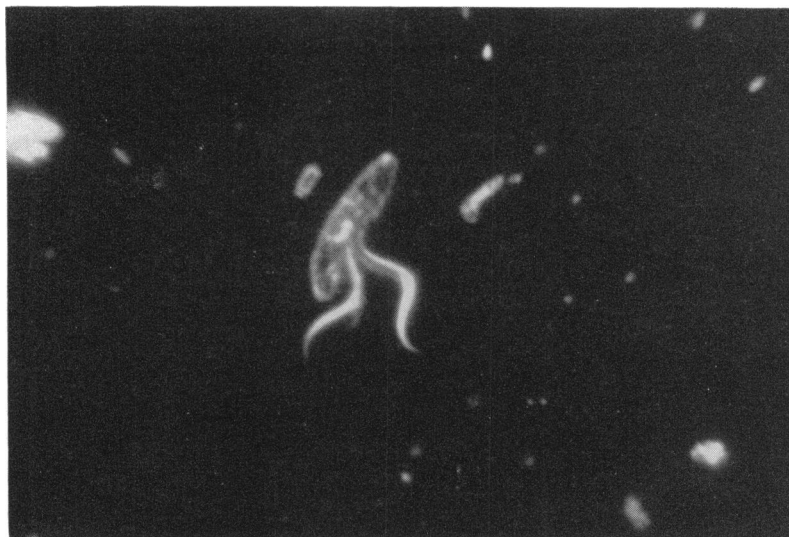


Figure 14. Darkfield photomicrograph of *Selenomonas palpitans* from guinea pig ileum, photographed at $\times 1000$.

thioglycolate broth cultures appeared as motile, slightly curved rods averaging 6 by 1 μ with rounded or bluntly tapered ends. After several months of subculture, some strains showed diminution in size to as little as 3 by 0.5 μ . S-curved forms with an average length of 10 to 12 μ were sometimes seen. Flexibility was not observed. Flagella were usually visible, sometimes trailing behind the cell and sometimes preceding it in the direction of movement. Single curved cells usually appeared to have one flagellum, i. e., a tuft of coalesced flagella. The point of origin was frequently subterminal on the concave side or in the center of the concave side (figures 1 to 4), but organisms with the flagellum emanating from other positions—terminal or from the convex side, were also observed (figures 5 and 6). Two flagella in random arrangement were sometimes seen (figures 7 and 8). Often all these arrangements could be seen in a single field (figure 9).

The random distribution of flagella was evident also in electron photomicrographs. A group of flagella sometimes emanated from the concave surface (figure 10). Sometimes the flagella appeared only on the convex surface (figure 11). Sometimes they were clearly terminal (figure 12) or subterminal (figure 13). The two strains of *Selenomonas ruminantium* had the same cell morphology and variety of flagellar arrangements as found in *Spirillum sputigenum*.

All attempts to cultivate *Selenomonas palpitans* were unsuccessful. By darkfield it was very much larger than *Spirillum sputigenum*. Its mean dimensions measured against polystyrene particles were 2.5 by 9 μ . Some cells were as large as 3 by 15 μ , appeared to have a complex internal structure, and carried one or two very broad bands of flagella (figure 14).

DISCUSSION

The 11 strains of *Spirillum sputigenum* used in this study were alike in their morphological, cultural, and physiological characteristics and conformed in every respect to the earlier description of 2 strains (Macdonald, 1953). They are clearly representative of a single species. The toxicity of this organism for rabbits has been described in more detail previously (*op. cit.*). It was associated with the culture supernatant, and the effect was thought to be anaphylactoid in character. Washed cells were without effect.

The present findings in respect to the arrangement of flagella indicate clearly that the concavity is not the only point of origin. The flagella were found to originate either singly or as tufts from virtually any position on the cell. This observation is not new. Hoffman and von Pro-wazek (1906) showed drawings of *Spirillum sputigenum* with flagella originating terminally and on the concave side. Muhlens (1909) and Repaci (1912) showed photomicrographs of this organism

with flagella irregularly distributed. Veillon and Repaci (1912) claimed peritrichous flagellation for *Spirillum crassum*, an organism subsequently identified as *Spirillum sputigenum*. Similar findings have been reported by Seguin (1928) and Seguin and Boisvert (1942). *Selenomonas ruminantium* also appears to have randomly distributed flagella as reported by Bryant (1956) and confirmed in the present study.

Nevertheless it is perhaps improper to refer to the arrangement of flagella in these organisms as peritrichous which is usually defined as meaning that flagella project from the whole body of the cell. The fact is that the flagella originate in an apparently random fashion either singly or as tufts from any point on the cell circumference.

These observations cast doubt on the wisdom of classifying these organisms in a new genus, *Selenomonas*, as has been done in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957). Flagellation from the concavity was the sole reason for recognizing the genus. It would seem wiser to include the oral species and the rumen organism (distinguished from the former on the basis of H₂S production) in the genus *Spirillum* under the names *Spirillum sputigenum* and *Spirillum ruminantium*.

The third organism, *Selenomonas palpitans*, should be recognized only in the Index Bergeyana for the present. Its size, apparent complexity, and its flagellation suggest that it may well be a protozoan entirely unrelated to the oral or rumen species.

SUMMARY

Eleven strains of *Spirillum sputigenum* have been described. They were found comparable in every respect to two previously described strains.

The flagellation of *Spirillum sputigenum*, *Selenomonas ruminantium*, and *Selenomonas palpitans* was examined by darkfield. The former two organisms were also examined by electron microscopy. The flagella in *Spirillum sputigenum* and *Selenomonas ruminantium* originated in a random fashion, singly or as tufts, from any point on the circumference. The flagella of *Selenomonas palpitans* appeared in broad bands emanating from the concave surface. The validity of the genus *Selenomonas* is questioned and the proposal made that the organisms classified in *Bergey's Manual of Determinative Bacteriology*

as *Selenomonas sputigena* and *Selenomonas ruminantium* be classified with the genus *Spirillum* under the names *Spirillum sputigenum* and *Spirillum ruminantium*. It is suggested that *Selenomonas palpitans* may be a protozoan and that, for the present, it be not recognized as a bacterial species.

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